Remodeling muscles with calcineurin

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Summary

Ca^{2+} signaling plays a central role in hypertrophic growth of cardiac and skeletal muscle in response to mechanical load and a variety of signals. However, the m chanisms whereby alterations in Ca^{2+} in the cytoplasm activate the hypertrophic response and result in longterm changes in muscle gene expression are unclear. The Ca^{2+}, calmodulin-dependent protein phosphatase calcineurin has been proposed to control cardiac and skeletal muscle hypertrophy by acting as a Ca^{2+} sensor that couples prolonged changes in Ca^{2+} lev ls to reprogramming of muscle gene expression. Calcineurin also controls the contractile and metabolic properties of skeletal muscle by activating the slow muscle fiber-specific gene program, which is dependent on Ca^{2+} signaling. Transcription factors of the NFAT and MEF2 families serve as endpoints for the signaling pathways whereby calcineurin controls muscle hypertrophy and fiber-type. We consider these findings in the c ntext of a model for Ca^{2+}-regulated gene expression in muscle cells and discuss potential implications of th se findings for pharmacologic modification of cardiac and skeletal muscle function. *BioEssays* 22:510–519, 2000. © 2000 John Wiley & Sons, Inc.

Introduction

Skeletal and cardiac muscle undergo hypertrophic growth in response to increased workload and a variety of intrinsic and extrinsic stimuli. Recent studies have begun to reveal the intracellular signaling pathways and transcription factors that control muscle hypertrophy and suggest that skeletal and cardiac muscle share common hypertrophic pathways. There is substantial evidence that Ca^{2+} signaling plays a pivotal role in regulating muscle cell hypertrophy. Thus, the discovery that the Ca^{2+}, calmodulin-dependent protein phosphatase calcineurin acts as a sensor of sustained elevations in intracellular Ca^{2+} concentration and triggers the transcriptional cascades that govern muscle growth, contractility, and metabolism has generated intense interest.[1–7]

Calcineurin has been shown to transduce hypertrophic signals leading to cardiac growth[1,2] and to mediate the hypertrophic effects of insulin-like growth factor1 (IGF1) on skeletal muscle.[3,4] In addition, calcineurin signaling has been found to control gene regulatory programs that are distinct among different subtypes of skeletal muscles,[5,6] and the ability of skeletal muscle cells to regenerate.[7] While these findings provide exciting new insights into the molecular mechanisms underlying muscle cell growth and fiber-type specificity, they also raise many interesting questions about the sensing mechanisms that enable a specific Ca^{2+} signal to be detected in the midst of the enormous fluctuations in Ca^{2+} levels that accompany each cycle of muscle contraction and relaxation. Since calcineurin can be specifically inhibited by drugs already approved for use in humans, these studies also suggest potential pharmacologic approaches for modifying muscle function through regulation of calcineurin activity. Because hypertrophic growth of cardiac muscle can be life-threatening, whereas hypertrophic growth of skeletal muscle can have obvious benefits, strategies are necessary for selectively targeting drugs that stimulate or inhibit muscle growth to one muscle type or the other. In this review, we consider these issues in the context of a model for Ca^{2+}/calcineurin signaling in skeletal and cardiac muscle cells.

Signaling by calcineurin/NFAT

Calcineurin, also called protein phosphatase 2B, is a serine, threonine protein phosphatase localized to the cytoplasm.[8,9] The enzyme is ubiquitous, but is present at about 10-fold higher concentrations in brain and muscle than in other cell types. Calcineurin exists as a heterodimer, composed of a 59 kDa catalytic subunit (CNA) that contains a calmodulin-binding domain and an autoinhibitory region, and a 19 kDa Ca^{2+}-binding regulatory subunit (CNB). Both subunits are essential for catalytic activity of the holoenzyme, but the CNA subunit can be activated constitutively, without a requirement for Ca^{2+} and calmodulin, by deletion of the carboxyl-terminal regulatory region.[10]

Calcineurin is tightly regulated by intracellular Ca^{2+} concentration and was first shown to b activated in T cells in respons to sustained elevation in intracellular Ca^{2+}

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Abbreviations: CaMKIV, Calmodulin-dependent protein kinase; CNA, Calcineurin A, CNB, Calcineurin B; CsA, Cyclosporin A; GSK3, Glycogen synthase-3 kinase; IGF, Insulin-like growth factor; JNK, Jun-amino-terminal kinase; MEF2, Myocyte-specific enhancer factor 2; NFAT, Nuclear factor of activated T-cells.

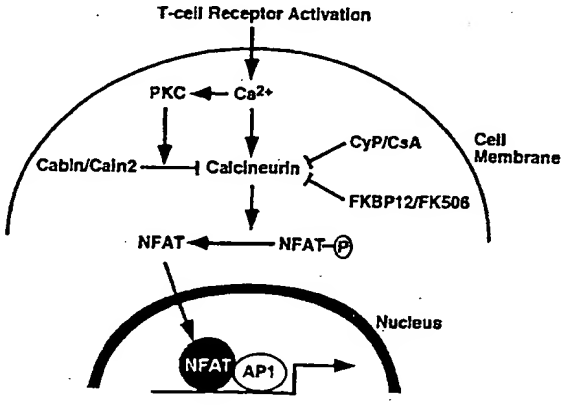

Figure 1. Calcineurin signaling in T cells. T-cell receptor activation results in activation of calcineurin, which dephosphorylates NFAT transcription factors, enabling them to translocate to the nucleus. NFAT proteins stimulate transcription in association with other transcription factors, including AP-1, activated by costimulatory signaling pathways. Cabin/Cain inhibit calcineurin activity. CsA and FK506 bind cyclophilin (CyP) and FKBP12, respectively, to form complexes that inhibit calcineurin activity.

(Fig. 1).[11] Activated calcineurin binds and dephosphorylates members of the NFAT family of transcription factors, resulting in their translocation to the nucleus where they interact with the transcription factor AP-1 and activate transcription of cytokine genes required for T-cell activation and proliferation.[8,9] Calcineurin activity is inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK-506, which bind the cytoplasmic receptor proteins, cyclophilin A and FKBP 12, respectively, forming complexes that interact with calcineurin, and thereby prevent activation of calcineurin-dependent genes and T-cell activation.[12]

Responsiveness to calcineurin can also be inhibited by the cytoplasmic calcineurin-binding proteins Cabin/Cain[13,14] or MCIP1 and MCIP2.[15] Activation of protein kinase C stimulates association of Cabin with calcineurin, providing a negative feedback mechanism to inhibit calcineurin signaling in response to T-cell activation.[14] Intriguingly, Cabin protein is expressed abundantly in brain, but only at low levels in skeletal muscle and heart,[13] while MCIP1 and MCIP2 are most abundant in striated muscle tissues.[15] This cell-type selective expression of proteins that inhibit calcineurin activity raises the possibility of developing drugs that act in a tissue-specific manner to control the calcineurin pathway.

Five NFAT genes have been identified in vertebrates (NFATc1/NFATc/NFAT2; NFATc2/NFATp/NFAT1; NFATc3/NFAT4/NFATx; NFATc4/NFAT3; NFAT5). All members of the NFAT family except NFAT5 are activated by calcineurin.[8,9,16,17] Although studied primarily in T cells, NFAT proteins are also expressed in other cell types and NFAT2 and NFAT4 isoforms are especially abundant in skeletal muscle.[17]

NFAT transcription factors are subject to a dynamic and reversible cycle of phosphorylation/dephosphorylation that controls their localization to the nucleus and activation of NFAT-dependent genes.[8,9] In unstimulated cells, NFAT proteins are maintained in the cytoplasm by phosphorylation of a serine-rich region near their amino-termini.[11] Dephosphorylation of these serines by calcineurin unmasks two nuclear localization sequences, resulting in rapid nuclear translocation.[18] Once in the nucleus, dephosphorylated NFATc1/NFATc proteins have been shown to be rephosphorylated by one or more priming kinases that create a phospho-recognition site for glycogen synthase-3 kinase (GSK3), resulting in their translocation back to the cytoplasm and termination of calcineurin-regulated transcription.[18] NFATc3/NFAT4 has also been shown to be phosphorylated in the nucleus by Jun-amino-terminal kinase (JNK) and the combination of MEKK1 and casein kinase-1a.[19,20] It has been proposed that phosphorylation stimulates nuclear export of NFATs by exposing a nuclear export sequence or regulating the nuclear import sequence. The plethora of kinases and phosphatases that act on NFAT proteins provides multiple potential targets for pharmacologic manipulation of NFAT-dependent cellular responses.

In addition to activating NFAT-dependent genes, calcineurin has also been shown to activate the MADS-box transcription factor MEF2 through a post-translational mechanism that remains to be defined.[21] MEF2 activates various immediate early genes, including *c-jun*, as well as many muscle-specific genes (reviewed in 22), and is a likely target for calcineurin signaling in muscle cells (see below).

Control of cardiac hypertrophy by calcineurin

Hypertrophic growth is an adaptive response of the heart to a variety of intrinsic and extrinsic stimuli and is important for increasing cardiac output. There are numerous parallels between the signaling systems involved in T-cell activation and those involved in stress-responsiveness of cardiac muscle cells. In response to hypertrophic signals, cardiomyocytes activate a cellular response characterized by an increase in cell size, sarcomere assembly, induction of fetal cardiac genes and repression of genes encoding the corresponding adult isoforms.[23,24] The involvement of Ca^{2+}-dependent signal transduction pathways in cardiac hypertrophy has been extensively documented, but which of these pathways actually function in vivo and how they are coupled to cardiac transcription factors to activate fetal cardiac genes in the adult heart are important unanswered questions.

The cardiac-restricted zinc finger transcription factor GATA4 is among the transcription factors shown to activate

hypertrophic-responsive genes in cardiomyocytes.[25,26] In a search for GATA4 cofactors, it was discovered that NFAT3 interacted with the second zinc finger of GATA4 to form a complex that activates transcription of the *b-type natriuretic peptide* gene, a well-characterized marker of the hypertrophic response.[1] The discovery that a downstream component of the calcineurin signaling pathway interacted directly with GATA4, suggested the possibility that activated calcineurin might transduce hypertrophic signals. Consistent with this notion, treatment of primary cardiomyocytes with CsA prevented hypertrophy in response to the hypertrophic agonists angiotensin II and phenylephrine,[1] both of which are known to activate intracellular Ca^{2+} signaling. Moreover, transgenic mice that expressed an activated form of calcineurin in the heart developed dramatic cardiac enlargement that progressed to dilated cardiomyopathy, heart failure and sudden death. Expression in the heart of an activated form of NFAT3 lacking the amino-terminal phosphorylation sites also resulted in hypertrophy, supporting the conclusion that NFAT acts as a transcriptional mediator of calcineurin signaling in the heart and is sufficient to activate the hypertrophic program in the absence of other upstream elements in a hypertrophic signaling pathway. A model that can account for the above findings is shown in Fig. 2.

The ability of activated NFAT3 to induce cardiac hypertrophy distinguishes this transcriptional response from NFAT-dependent responses in T cells, in which calcineurin-NFAT signaling cooperates with costimulatory pathways to converge with AP-1, composed of Fos and Jun heterodimers, on downstream target genes.[27] Either NFAT does not require AP-1 for transcriptional activity in cardiomyocytes, possibly because it can synergize with GATA4, or AP-1 is already activated in cardiomyocytes. Of note, AP-1 has also been shown to cooperate with GATA4 to activate hypertrophic-responsive genes in the heart in response to pressure overload.[25,26] A complete understanding of the mechanism whereby activated NFAT3 induces hypertrophy will require identification of the key target genes in this transcriptional pathway.

While NFAT is the best-characterized transcriptional target for calcineurin signaling, it is also possible that other transcription factors, such as MEF2,[22] mediate the effects of calcineurin in cardiac muscle. There could also be transcriptional repressors controlled by calcineurin that influence expression of hypertrophic-responsive genes.

Is calcineurin a common mediator of diverse hypertrophic stimuli?

The discovery of a calcineurin signaling pathway for cardiac hypertrophy raises the possibility that aspects of this disease might be ameliorated by systemic delivery of calcineurin inhibitors. This suggestion is supported by the finding that hypertrophy in transgenic mice expressing activated calcineurin in the heart could be prevented by high doses of CsA.[1] Notably, calcineurin activity has been shown to be elevated several fold in cardiac extracts from heart failure patients,[28] though this finding has recently been challenged.[29]

It is estimated that 1 in 500 people develops cardiac hypertrophy due to mutations in cardiac contractile or cytoskeletal proteins that perturb contractility.[30] There is evidence that cardiomyocytes harboring such mutations exhibit alterations in Ca^{2+} sensitivity,[31] but the signaling mechanism that senses perturbations in contractility and conveys signals to the nucleus to activate the hypertrophic response is unknown. The possibility that some such mutations might cause hypertrophy through calcineurin activation is supported by the finding that three lines of

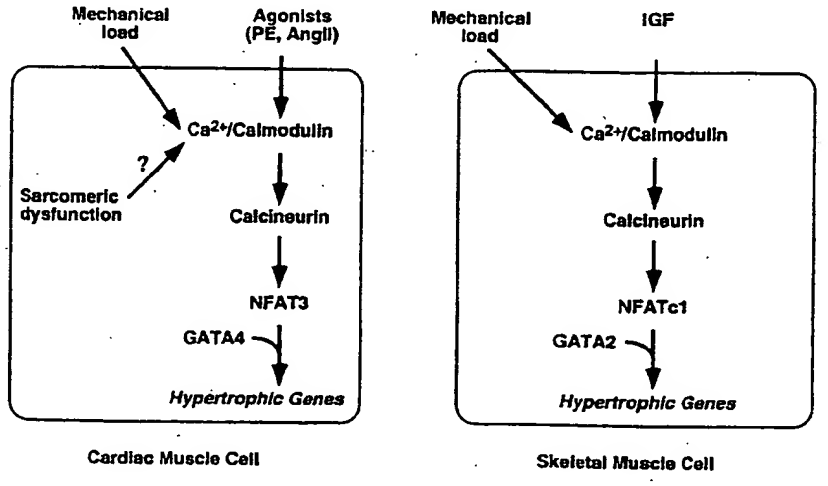

Figure 2. Calcineurin-dependent pathways for hypertrophic signaling in cardiac and skeletal muscle cells. Calcineurin has been shown to mediate the effects of the hypertrophic agonists PE and angiotensin II in cardiomyocytes and IGF-1 in skeletal myocytes. Mechanical load has also been shown to induce hypertrophy by calcineurin signaling. Different members of the GATA and NFAT families of transcription factors appear to act downstream of calcineurin in cardiac and skeletal myocytes.

transgenic mice predisposed to developing hypertrophy due to altered contractile function can be prevented from developing cardiomyopathy by treatment with CsA or FK-506.[2] Cardiac-specific overexpression of the actin-capping molecule tropomodulin, beta-tropomyosin, or a nonphosphorylatable form of ventricular myosin light chain-2 results in myofibrillar disarray and cardiomyopathy. Calcineurin enzyme activity is elevated in the hearts of these transgenic mice and treatment with CsA prevents cardiac disease.[2] Whether other forms of hypertrophy arising from altered contractility also involve calcineurin activation remains to be determined.

CsA administration has also been shown to reduce, but not entirely prevent, hypertrophy in mice that express activated Gq in the heart,[32] suggesting that calcineurin activation is one of multiple downstream pathways activated by Gq. However, there are also transgenic mouse models of hypertrophy that are not rescued by CsA administration.[2] This demonstrates specificity in the response of certain transgenic lines and also indicates the existence of calcineurin-independent mechanisms for hypertrophic growth of the heart.

Currently, there is disagreement concerning the potential importance of calcineurin for cardiac hypertrophy in response to pressure overload following aortic constriction.[33,34] Some studies[2,35] have shown that pressure-overload hypertrophy can be completely prevented in rats by CsA, whereas others have found only partial[36,37] or no diminution[38–40] in the hypertrophic response. It has also been reported that CsA fails to diminish hypertrophy in spontaneously hypertensive rats, a well characterized model for hypertrophic growth.[36] Moreover, it is well established that human patients maintained on CsA[41] can develop cardiac hypertrophy.

While the exact basis for these opposing conclusions remains to be resolved, several points should be considered. First, it is unclear whether systemic administration of CsA inhibits sufficient calcineurin activity in the heart to block the calcineurin/NFAT pathway, particularly given the high levels of calcineurin in the heart. Second, there is wide variability in the methods used for aortic constriction in rodents and it is well established that the position on the aorta and severity of the constriction can result in dramatically different responses of the heart, as well as activation of different signaling systems.[42] Thus, it is perhaps not surprising that different studi s yield different results. Third, aortic constriction is often accompanied by a high degree of mortality that may select for genetic variants that exhibit distinct responses to the pressure-overload stimulus.

What is clear is that high doses of CsA are toxic to rodents and result in multiple secondary effects that can complicate interpretation of results. Consequently, resolution of the debate concerning the potential importance of calcineurin signaling in pressure-overload hypertrophy will requir genetic ablation of specific components of the pathway.

Control of skeletal muscle differentiation and regeneration by calcineurin

The question of whether calcineurin-NFAT signaling plays similar roles in cardiac and skeletal muscle is of particular interest, given the high levels of expression of certain NFAT isoforms in skeletal muscle.[7,17]

Skeletal muscle differentiation involves irreversible withdrawal of myoblasts from the cell cycle, fusion to form multinucleate myotubes, and transcriptional activation of muscle-specific genes. Abbott et al.[7] reported that CsA inhibits myoblast differentiation in vitro, suggesting that calcineurin activity is essential for activation of the muscle differentiation program. NFAT isoforms were also shown to be differentially translocated from the cytoplasm to the nucleus at different stages of myoblast differentiation, suggesting that they respond to distinct signals in the cytoplasm.[7]

Damage of adult skeletal muscle results in activation of quiescent myogenic satellite cells, adjacent to muscle fibers, that fuse with pre-existing myofibers resulting in muscle repair. CsA prevents muscle regeneration in response to damage, further suggesting an important role for calcineurin signaling in muscle development in vivo.[7] This may also begin to explain why transplant patients treated with CsA show severe skeletal muscle weakness.[43]

Control of skeletal muscle hypertrophy by calcineurin

IGFs are potent inducers of skeletal muscle hypertrophy in vivo and in vitro, acting by stimulating expression of L-type Ca^{2+} channels, which increase near-membrane Ca^{2+} concentrations.[44,45] Evidence for an essential role of calcineurin activation in IGF1-mediated skeletal myocyte hypertrophy has come from the finding that expression of an IGF1 expression vector in cultured rat myoblasts or exposure to IGF1 results in dramatic hypertrophy accompanied by upregulation of calcineurin expression and its nuclear localization.[3,4] Conversely, CsA blocks hypertrophy in response to IGF1.

Both IGF1 and activated calcineurin induce expression of GATA2 in skeletal muscle, which associates with calcineurin and a nonphosphorylated form of NFATcl.[3] These findings suggest that cardiac and skeletal muscle cells share aspects of a common hypertrophic signaling system mediated by calcineurin, NFAT and GATA transcription factors (Fig. 2).

Intracellular Ca^{2+} levels are elevated in skeletal muscle fibers in response to chronic overload,[46] and recently, it was shown that treatment of rats with CsA and FK-506 prevented th doubling in muscle mass and increas in slow muscle fibers normally associated with functional ov rload in vivo,[6] consistent with the possibility that calcineurin signaling

mediates exercise-induced muscle growth, as well as fiber-type transformation. The involvement of calcineurin in the control of both myofiber size and specialization is supported by other analyses of humans or laboratory animals treated with CsA. Effects of pharmacologic inhibition of calcineurin to reduce muscle mass, to reduce expression of enzymes of oxidative metabolism and to reduce the proportion of Type I fibers have been described.[47–51] In human transplant recipients receiving calcineurin antagonist drugs, it is difficult to distinguish the direct consequences on skeletal muscle properties from the consequences of physical inactivity resulting from the underlying disease.

Myostatin, a member of the TGF-β superfamily expressed specifically in skeletal muscle, appears to act as an inhibitor of skeletal muscle hypertrophy, since strains of livestock or transgenic mice bearing mutations in the myostatin gene exhibit massive increases in muscle size.[52] It will be of interest to understand whether or how myostatin signaling impinges on the calcineurin/NFAT signaling pathway.

Diversity and plasticity of skeletal muscle fibers

Skeletal myofibers exhibit highly specialized characteristics with respect to size, metabolism and contractile function. Slow-twitch muscles are utilized primarily for antigravity functions or for sustained locomotor activity and consist largely of myofibers that are rich in mitochondria and enzymes of oxidative metabolism. Slow myofibers express specialized forms of myosin and other sarcomeric proteins that are highly efficient in the conversion of chemical energy into contractile work. Fast-twitch muscles are called upon infrequently for swift and powerful bursts of contractile work. Fast myofibers express alternative forms of sarcomeric proteins that generate force rapidly, but less efficiently with respect to energy cost, and a subset of fast fibers (type II) ar enriched in enzymes of glycosis. Fast fibers of a different subset (type IIa) resemble type I fibers with respect to high expression of proteins of oxidative metabolism such as myoglobin and mitochondrial enzymes of oxidative phosphorylation.

Specialized programs of gene expression that define distinctive myofiber subtypes are established during fetal development, even prior to the formation of neuromuscular junctions, and can be maintained clonally in isolated myoblasts propagated in primary culture.[53] These observations indicate the existence of multiple sublineages of myogenic precursor cells that can generate phenotypic diversity among mature myofibers. Following innervation, however, the motor nerve exerts influences over gene expression in skeletal myofibers that can override any preprogramming that was established during fetal development, resulting in interconversion of fiber types by varying the frequency that the motor nerve fires.[54–57]

How ar signals from the motor nerve sensed and transduced?

The molecular signaling mechanisms by which variations in motor nerve activity are sensed and transduced to control the expression of specific genes that define myofiber subtypes or that regulate myofiber size and number remain to be identified. At least three general sensing mechanisms can be envisioned: (1) activation of cell surface receptors by extracellular signaling molecules released from the motor nerve or from other cell types as a consequence of neuromuscular activation, (2) direct sensing of mechanical forces generated by loading conditions, and (3) sensing of intracellular metabolite concentrations that change as a consequence of muscle contraction.

Motor nerve stimulation releases acetylcholine, which binds to specific receptors localized to the motor end plate within the neuromuscular junction. Agonist binding to nicotinic acetylcholine receptors triggers the familiar cascade of events leading to muscle contraction: myofiber depolarization and release of Ca^{2+} stored within the sarcoplasmic reticulum, and Ca^{2+}-dependent sliding of actin filaments within the sarcomere driven by the motion of myosin heads.

Loading conditions exert a powerful influence on muscle size and on expression of specialized protein isoforms in skeletal myofibers, implying that mechanical forces can be sensed, directly or indirectly, by proteins that control signaling cascades linked to gene expression. Pathways dependent upon focal adhesion kinase have been implicated in myocyte hypertrophy,[58,59] and metabolite-responsive signaling mechanisms[60,61] also may contribute to muscle plasticity in response to changes in contractile activity. This review, however, focuses primarily on Ca^{2+} as a first messenger.

Ca^{2+} as a messenger linking neural activity to gene expression in skeletal muscles

Since motoneuron activity exerts such a dominant effect on myofiber phenotype, perhaps neural regulation of gene expression in this cell type is driven by the same first messenger involved in neuromuscular coupling, namely intracellular Ca^{2+}. As shown in Fig. 3, different patterns of motoneuron activation that stimulate distinctive programs of gene expression (e.g. slow oxidative versus fast glycolytic myofibers) generate different Ca^{2+} waveforms. Motoneurons that innervate slow myofibers fire on an almost continuous basis, such that cytosolic Ca^{2+} oscillates in a concentration range between 100 and 300 nM.[62,63] In contrast, motoneurons that innervate fast glycolytic myofibers fire only sporadically. In the long periods between contractions, intracellular Ca^{2+} concentrations are held below 50 nM, but incr ase transiently to th range of 1 μM upon neuromuscular activation.[64] As noted previously,

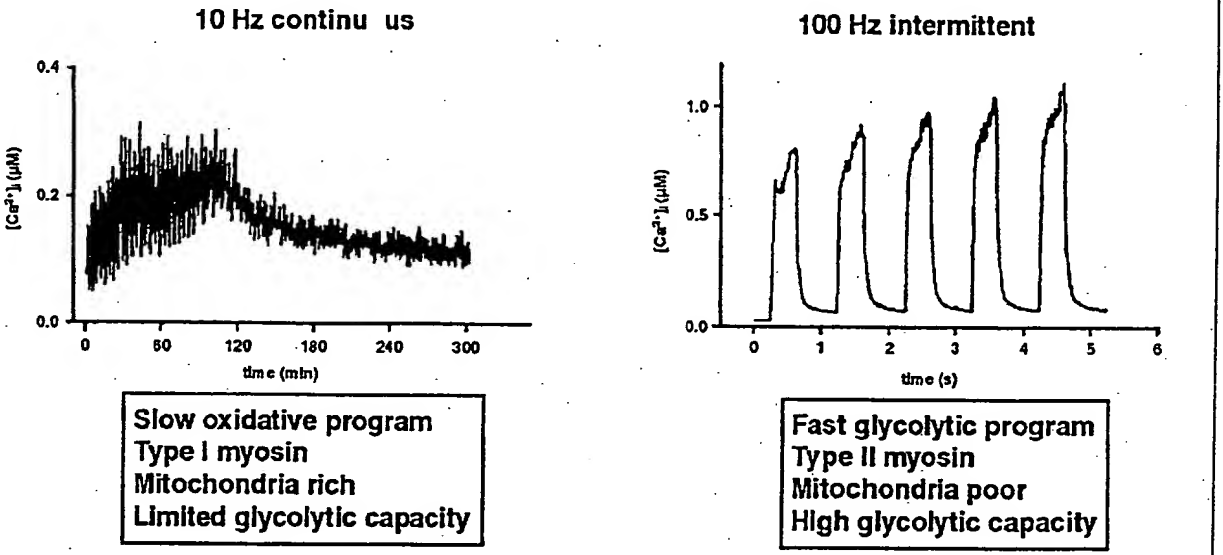

Figure 3. Different patterns of motor neuron firing lead to distinctive waveforms of oscillations in intracellular calcium ($[Ca^{2+}]$ i), and determine skeletal muscle fiber type. Continuous stimulation at 10Hz (left panel) leads to sustained elevations of $[Ca^{2+}]$i in a range from 100 to 300 nM that are characteristic of slow fibers. Intermittent nerve stimulation at higher frequencies (100Hz) produces large but transient oscillations in $[Ca^{2+}]$i that reach as high as 1000 nM, but are maintained at resting levels around 50 nM in a manner characteristic of fast fibers. Varying the frequency of neural stimulation will transform one fiber type into another.

cross-innervation or electrical pacing experiments that alter the rate and pattern of neuromuscular activation, and therefore the temporal patterns of intracellular Ca^{2+} concentrations, promote fiber-type transformation.[54–57,65] Exercise training regimens that involve tonic muscle contractions for as little as 30 to 60 minutes daily are sufficient to evoke changes in myofiber phenotype with respect to proteins of oxidative metabolism such as myoglobin and mitochondrial enzymes,[66,67] but longer periods of neural activity are necessary to promote fast-to-slow myofiber transformation.

Based on an ability to discriminate among different Ca^{2+} waveforms,[68] calcineurin is an attractive candidate to function as a nodal point in molecular pathways by which changes in neural stimulation and contractile activity are sensed by skeletal myofibers, and transduced to promote long-term changes in gene expression leading to myofiber specialization and hypertrophic growth (Fig. 4). Several lines of experimental evidence support the notion that calcineurin indeed functions in this capacity. The components of Ca^{2+}-regulated, calcineurin-dependent signal transduction pathways that have been defined in other cell types also are expressed and available in skeletal muscle. Activated calcineurin stimulates transcription directed by well-defined muscle-specific enhancers from genes expressed selectively in slow or oxidative myofiber subtypes such as *myoglobin* (oxidative fiber-specific) and *troponin I slow* (slow fiber-specific).[5,69] Expression of a constitutively active form of calcineurin under the control of the muscle creatine kinase enhancer leads to an increased proportion of type I fibers within muscles of the hind limb of transgenic mice.[70] Conversely, administration of calcineurin antagonists to rats or mice blunts expression of genes that are preferentially transcribed in slow, oxidative myofibers.[5,6] Calcineurin regulates the function of NFAT and MEF2 transcription factors that act synergistically in skeletal myocytes.[5] In the case of MEF2, an activating effect of calcineurin is evident in the absence of DNA binding by NFAT or other heterologous DNA-binding proteins.[79] In the case of NFAT, DNA binding by MEF2 (or other co-activators) to sites adjacent to the NFAT recognition motif is required for calcineurin-stimulated transactivation. Within native enhancer elements from the *myoglobin* or *troponin I* slow genes, disruption of either NFAT- or MEF2-binding sites abrogates responsiveness to calcineurin in cultured myocytes,[5] and their selective expression within slow, oxidative myofibers in transgenic mice.[79] Other studies in transgenic mice show that enhancers lacking an identifiable NFAT element can be expressed selectively in slow fibers;[69,71] (Wu, H and Williams, RS, unpublished data), suggesting either that other effectors (.g. MEF2) can transduc calcineurin-dependent signals to the r l - vant downstream genes, or that calcineurin-independent

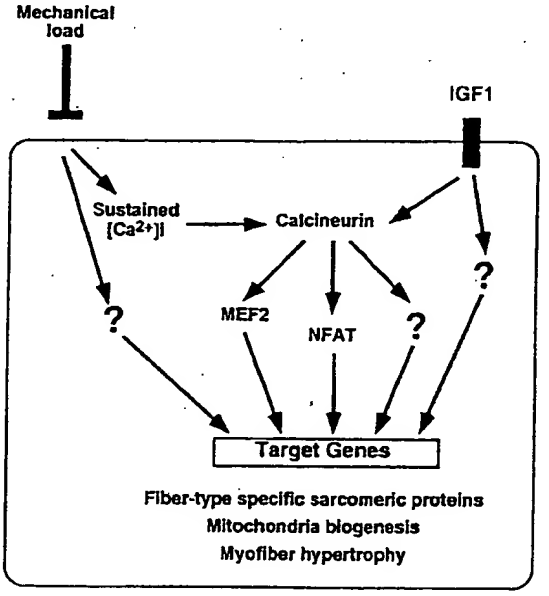

Figure 4. Signaling pathways implicated in skeletal muscle hypertrophy and slow fiber gene expression. Calcineurin acts downstream of motor nerve and the IGF receptor. Other possible pathways not yet defined are indicated by (?).

mechanisms are, at least under some conditions, sufficient to drive expression of slow fiber-specific genes.

Interestingly, skeletal myotubes made hypertrophic by IGF1 upregulate expression of enzymes of glycolytic, not oxidative, metabolism.[4] Both hypertrophy and this shift to a glycolytic phenotype are blocked by inhibitors of calcineurin. This finding suggests that specific downstream events that are triggered by calcineurin are modified on the basis of cross-talk with other signaling pathways (in this case, other signals triggered by IGF1) (Fig. 4).

C nverting Ca^{2+} signals to changes in gene expression

To explain fully how Ca^{2+} signaling can regulate long-term changes in gene expression in muscle, it is essential to und rstand how prolonged changes in Ca^{2+} can be distinguished from transient Ca^{2+} signaling as occurs during each phase of contraction/relaxation. Studies of the Ca^{2+}-dep ndence of NFAT activation have shown that NFAT is maintained in the nucleus only in response to prolonged, low-amplitude Ca^{2+} signals and is insensitive to high-amplitude Ca^{2+} transients.[68] The ability to discriminate between different types of Ca^{2+} signals reflects specificity in the kinases and phosphatases that act on different NFAT proteins and provides for specificity in responsiveness of different NFAT proteins to Ca^{2+} signaling. Further specificity in transcriptional responses to Ca^{2+} signaling may occur through differences in protein-protein interactions of different NFAT isoforms with several classes of co-activators (MEF2, GATA; or AP1), enabling them to activate different sets of downstream target genes.

Future questions

The proposition that calcium is a critical first messenger and calcineurin an important control point in regulatory pathways by which skeletal myofibers sense and respond to changes in neural stimulation and contractile activity has invigorated this area of research in muscle biology. Recent research in this area has established the importance of this general concept, but many questions remain.

What are the transcriptional targets for calcium-regulated and calcineurin-dependent signaling pathways in cardiac and skeletal muscles that control hypertrophic growth?

So far, specific genes that have been shown most clearly to respond to hypertrophic signaling pathways appear to represent markers of the process, rather than encoding proteins that act directly as the terminal effectors of hypertrophy.

How are calcineurin-dependent events integrated with other signaling inputs to generate different phenotypic responses in cardiac and skeletal myocytes?

In the case of cardiac muscle, the interactions between calcineurin-independent mechanisms leading to hypertrophy and events dependent upon calcineurin signaling must be defined more fully. This issue assumes special importance with respect to potential clinical applications of new knowledge in this area. Pharmacological interdiction of calcineurin signaling to prevent heart failure in humans is an attractive concept, but this goal depends upon greater knowledge of the relative importance of different hypertrophic signaling cascades.

How do skeletal myofibers distinguish between signals that promote hypertrophic growth and those that drive fiber type-selective programs of gene expression?

Although these distinct features of skeletal muscle plasticity may occur in concert as a response to certain stimuli (e.g. hypertrophy and up-regulation of glycolytic enzymes in response to IGF1; hypertrophy and fast-to-slow fiber transformation in respons to functional overload by ablation of functionally antagonistic muscle groups), other stimuli evoke clearly separable adaptations (e.g. fast-to-slow fiber

transformation without hypertrophy as a response to 10 Hz motor nerve stimulation). If we accept the premise that calcineurin is an important control point in both categories of responses, then distinctions among transcriptional events triggered by calcineurin must be based on dose-response considerations (e.g. different thresholds for evoking hypertrophy versus fast-to-slow fiber transformation) and/or differences in the activity of parallel signal transduction pathways that modify calcineurin-generated transcriptional regulation. Variation in the relative abundance of specific myogenic determination proteins[72] or other transcription factors (MusTRD1)[73] has been suggested as a potential mechanism for controlling myofiber diversity during muscle development. Future studies should examine the mechanisms of how different myogenic sublineages are established and clonally maintained during development, and determine the degree to which activity-dependent mechanisms of gene regulation in adult myofibers employ signaling molecules involved in muscle development.

How can we manipulate the calcineurin pathway selectively in a specific cell type?

For the purposes of fundamental research, the use of cell-type-restricted promoters to drive expression of constitutively active or dominant negative forms of signaling proteins accomplishes this goal, and this approach has provided a great deal of useful information. Clinical applications of new knowledge that relates to calcineurin signaling in skeletal and cardiac muscles, however, would be facilitated by the availability of drugs that act selectively within each of these cell types. Currently available antagonists of calcineurin such as CsA and FK-506 are unsuitable for trials to prevent heart failure, because of toxic consequences relating to antagonism of calcineurin in lymphocytes, neurons and skeletal myocytes. Drug-induced activation of calcineurin signaling in skeletal muscle could find medical application to increase muscle mass or to alter metabolic properties of skeletal muscle for therapeutic benefit in several categories of human disease. However, such drugs would be useful only if targeted selectively to skeletal muscle, thereby avoiding unwanted consequences of calcineurin activation in other tissues (e.g. cardiac hypertrophy).

The identification of proteins that, unlike calcineurin itself, are expressed selectively in cardiac and/or skeletal muscles would provide potential opportunities for achieving tissue-restricted modulation of calcineurin signaling. The calcineurin antagonist proteins MCIP1 and MCIP2[15] are interesting in this regard. It also may be possible to exploit cell-type-dependent differences in specific isoforms of NFAT, MEF2 or oth r proteins that function either as effectors or modulators of calcineurin signaling in order to find small molecules that alter calcineurin-dependent responses in cardiac and skeletal muscles.

Finally, while we have focused on the possible transcriptional targets for calcineurin signaling in muscle cells, it is worth noting that calcineurin has other substrates that play important roles in regulating intracellular Ca^{2+} handling and therefore have the potential to profoundly alter muscle function. In yeast, calcineurin regulates Ca^{2+} homeostasis and responsiveness to ionic stress by controlling expression of genes encoding Ca^{2+} and Na^{+} ATPases via the zinc finger transcription factor CRZ1/TCN1.[74–76] Similarly, calcineurin regulates Ca^{2+} fluxes in mammalian cells by associating with the IP3 and ryanodine receptors.[77,78] The extent to which these calcineurin substrates influence muscle growth and function through modulation of Ca^{2+} handling remains to be determined.

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